Plant Physiol. (1996) 112: 821–832

Severity of Mutant Phenotype in a Series of Chlorophyll-Deficient Wheat Mutants Depends on Light Intensity and the Severity of the Block in Chlorophyll Synthesis[1]

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Analyses of a series of allelic *chlorina* mutants of wheat (*Triticum aestivum* L.), which have partial blocks in chlorophyll (Chl) synthesis and, therefore, a limited Chl supply, reinforce the principle that Chl is required for the stable accumulation of Chl-binding proteins and that only reaction centers accumulate when the supply of Chl is severely limited. Depending on the rate of Chl accumulation (determined by the severity of the mutation) and on the rate of turnover of Chl and its precursors (determined by the environment in which the plant is grown), the mutants each reach an equilibrium of Chl synthesis and degradation. Together these mutants generate a spectrum of phenotypes. Under the harshest conditions (high illumination), plants with moderate blocks in Chl synthesis have membranes with very little Chl and Chl-proteins and membrane stacks resembling the thylakoids of the lethal *xantha* mutants of barley grown at low to medium light intensities (which have more severe blocks). In contrast, when grown under low-light conditions the same plants with moderate blocks have thylakoids resembling those of the wild type. The wide range of phenotypes of Chl *b*-deficient mutants has historically produced more confusion than enlightenment, but incomparable growth conditions can now explain the discrepancies reported in the literature.

It has been well documented that the thylakoids of so-called "shade plants" have larger LHCs and photosynthetic units than "sun plants" (800 Chl molecules/photosynthetic unit versus 200–500 Chl molecules/photosynthetic unit; and a Chl *a*/*b* ratio of <3.0 versus ≥3.0; Anderson, 1986; Anderson and Osmond, 1987; Melis, 1992). However, along with this observation come a great many unknowns. How does a plant determine the appropriate amounts of Chl and LHC proteins for its growth environment? What controls the biosynthesis of Chl *a* versus Chl *b*? What determines the choice between the synthesis of RCs versus LHC molecules under different growth conditions? To address these questions several laboratories have devoted much research to the study of mutants with reductions in Chl *b* because they have reduced photosynthetic unit sizes compared with wild-type plants (Ghirardi and Melis, 1988; Greene et al., 1988a, 1988b; Harrison et al., 1993) and could be considered as models of "sun plants." We have used such mutants to gain more direct insights into the mechanism(s) that control the development of the photosynthetic apparatus (Allen et al., 1988; Falbel and Staehelin, 1994; Falbel et al., 1994). Here we report how the rate of Chl synthesis affects the assembly of thylakoid membranes.

There are two classes of chloroplast mutants with reduced amounts of Chl *b*: one type has no Chl *b* at all, and the other is Chl *b*-deficient. The former type, Chl *b*-less mutants, such as barley *chlorina f2* (Bellemare et al., 1982) and *Arabidopsis thaliana ch1* (Murray and Kohorn, 1991), synthesizes no Chl *b* regardless of the light condition. Since this type produces nearly normal amounts of Chl *a*, it is generally assumed that primary lesions affect the biosynthesis of Chl *b* directly. Mutants with no Chl *b* typically fail to accumulate significant quantities of Chl *a*/*b*-binding LHCs under any growth condition (Harrison et al., 1993). Their stacked membrane regions have altered ultrastructure and adhesion properties compared with the wild type (Staehelin, 1986). In this paper we do not deal with the Chi *b*-less mutants because they affect the accumulation of Chl *b* and not Chl *a*, and the nature of the ultrastructural changes clearly puts these mutants in a different class than the Chl *b*-deficient mutants.

The focus of this study is the Chl *b*-deficient mutants, which also produce significantly less Chl *a* than wild-type plants. In addition, the total amount of Chl and the Chl *a*/*b* ratio vary greatly in response to changes in the light environment (similar to sun and shade plants) and to changes in temperature (Yang et al., 1990; Markwell and Osterman, 1992). Concomitant light- and temperature-dependent changes in LHCs, grana stacks, and general organization of the thylakoids have also been observed, with losses in peripheral LHCs in general paralleling reductions in the size and number of grana stacks (Greene et al., 1988a, 1988b; Knoetzel and Simpson, 1991). However, the pleio-

[1] This work was supported by a Howard Hughes Medical Institute Predoctoral Fellowship to T.G.F. and National Institutes of Health grant no. GM22912 to L.A.S.

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Abbreviations: BBY, Berthold, Babcock, and Yocum PS11A-enriched preparation; Chl, chlorophyll; CP, chlorophyll-protein; LHC, light-harvesting antenna complex; RC, reaction center.

tropic nature of the responses and the diversity of thylakoid morphologies is not yet well understood.

Many Chl *b*-deficient mutants have been identified, and in all instances in which the primary lesion of the mutants has been determined, the deficiency has been found to be due to a partial block in the Chl synthesis pathway (Falbel and Staehelin, 1994, 1996). Surprisingly, most are blocked at the same point in the pathway, at protoporphyrin IX, the branch point between Chl and heme synthesis. Based on this finding and on other developmental studies in which the supply of Chl *a* became limiting and RCs were assembled preferentially (Akoyunoglou et al., 1978; Akoyunoglou and Akoyunoglou, 1985), we have postulated that Chl *b* is made in significant amounts only when there is "leftover" Chl *a* because the RCs are no longer incorporating the majority of Chl *a*. In this way, the partial blocks in the main Chl synthesis pathway characteristic of the Chl *b*-deficiency mutations affect the biosynthesis of Chl *b* indirectly (Falbel and Staehelin, 1994, 1996). Because all Chl *b*-deficient mutants we have analyzed to date have blocks in the Chl synthesis pathway, we hypothesize that any partial block in Chl synthesis causes an indirect and amplified inhibition of Chl *b* synthesis because Chl *b* is made from leftover Chl *a* molecules.

The pleiotropic nature of the structural and biochemical phenotypes of the Chl *b*-deficient mutants can be explained by an extension of this theory. Because Chl *b* is needed for the stabilization of LHCs but not RCs (Bennett, 1981; Paulsen, 1995), the amplified inhibition of Chl *b* synthesis leads to smaller photosynthetic unit sizes and to the development of abnormal thylakoid membrane systems. In other words, the rate of accumulation of Chl in thylakoids determines the rate at which both Chl *b* and different types of Chl-binding proteins accumulate, as well as the steady-state equilibrium of RC and LHC assemblies in mature thylakoids.

To test this postulated relationship experimentally, we have manipulated the light conditions to up-regulate or down-regulate the rate of Chl accumulation in mutants with partially blocked Chl synthesis pathways. Our theory predicts that any high-light-induced slowdown in net Chl accumulation (e.g. photobleaching) should increase the severity of the mutant phenotype. Thus, if a "mild" mutant with a minor block in Chl synthesis is grown in low light its phenotype will be nearly normal, but if the same mutant is grown under high light its phenotype will be highly perturbed, comparable to a mutant with a major block in Chl synthesis grown in low light. In this report we use three Chl *b*-deficient mutants of a previously characterized series of hexaploid wheat mutants (*chlorina*-1, CD3, and *chlorina*-214; Falbel and Staehelin, 1994) to demonstrate that the apparent severity of the phenotype (Chl *a*/*b* ratio, size of RC-LHC assemblies, and thylakoid architecture) of a given mutant is indeed approximately proportional to the intensity of the light under which the mutant is grown. We also show that heterozygous tobacco (*Su*/+) and tomato (*Xa*-2/+) mutants, both of which are partially blocked at protoporphyrin IX, exhibit the same types of changes as the wheat mutants.

MATERIALS AND METHODS

Seed Sources

Wheat

Wild-type hexaploid wheat, *Triticum aestivum* strain ND496-25 (CD3 parent strain), and all mutant wheat strains (CD3, *chlorina*-1, *chlorina*-214, Driscoll's *chlorina*) were obtained from Dr. Murray Duysen (North Dakota State University, Fargo).

Tobacco

Nicotiana tabacum mutant *Su* was obtained from Dr. Verne Sisson (Tobacco Stock Center, U.S. Department of Agriculture Crops Research Laboratory, Oxford, NC). Wild-type seed was produced by selfing wild-type progeny from the selfed *Su* heterozygotes.

Tomato

Lycopersicon esculentum mutant *Xa*-2 was obtained from Dr. Charles Rick (University of California, Davis). Wild-type seed was produced by selfing wild-type progeny from the selfed *Xa*-2 heterozygotes.

Growth Conditions

Wild-type and mutant wheat to be prepared for electrophoresis were grown on moist vermiculite for 16 d under two different growth environments: "High light" (approximately 1600 μmol photons m^{-2} s^{-1}) was provided by natural light in a greenhouse supplemented by metal halide and sodium vapor lamps. "Low light" (approximately 100 μmol photons m^{-2} s^{-1}) was provided by cool-white fluorescent lamps. Wild-type and mutant wheat plants were grown under low-light conditions for approximately 3 weeks. Wild-type and mutant tobacco and tomato plants were grown under the same high-light and low-light growth conditions, but to generate sufficient leaf material the plants were grown for at least 4 weeks in a mixture of potting soil and vermiculite (1:1, v/v).

Wheat plants to be prepared for analysis by electron microscopy were grown on moist vermiculite for 7 d at three different light conditions. Samples grown at high light (approximately 600 μmol photons m^{-2} s^{-1}, metal halide lamps) and low light (approximately 30 μmol photons m^{-2} s^{-1}, cool-white fluorescent lamps) were taken from exactly the same plants that were analyzed for xanthophyll cycle carotenoids (Falbel et al., 1994). Plants grown at "medium light" were grown for 7 d under cool-white fluorescent light (approximately 150 μmol photons m^{-2} s^{-1}).

Chloroplast Preparations

Thylakoids were prepared from plants homogenized in 0.4 M sorbitol, 100 mM Tricine-KOH, pH 7.5, 10 mM NaCl, and 5 mM $MgCl_2$. After the sample was filtered through nylon mesh and centrifuged for 5 min at 4000*g*, the chloroplast pellet was lysed by resuspending in 5 mM Hepes-

KOH, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, and the thylakoids were pelleted by centrifugation (5 min, 4000*g*). Finally, thylakoids were washed in 5 mM Hepes-KOH, pH 7.5, 10 mM EDTA, centrifuged, and resuspended in the same buffer with 10% glycerol added. Samples were stored at −80°C in small aliquots.

Thylakoid samples that contained a lot of starch were further purified by flotation on Suc step gradients. After the Hepes-EDTA wash, thylakoids were resuspended in Hepes-EDTA in 1.8 M Suc. This layer was transferred to ultracentrifuge tubes (for either the Beckman SW27 or SW41 rotor) and overlayed with one-third volume of 1.3 M Suc, Hepes-EDTA and an equal volume of 0.5 M Suc, Hepes-EDTA. After 1.5 h of centrifugation in a swinging bucket rotor at 132,000g_{max}, starch-free thylakoids floated to the 1.3 M Suc layer. This layer was removed, washed by diluting with an excess of Hepes-EDTA, and recentrifuged. The starch-free thylakoids were resuspended in Hepes-EDTA-glycerol and stored in aliquots at −80°C.

PSI and PSII Isolations

Membrane preparations enriched for PSII, or BBY preparations, were made from wheat according to the method of Berthold et al. (1981) but without the second Triton X-100 treatment (as modified by Dunahay et al., 1984) and using Mes buffer at pH 6.1 according to the method of Ford and Evans (1983). The Triton X-100:Chl ratio required to obtain pure stacked membranes at a good yield varied somewhat (Falbel and Staehelin, 1992). It was determined empirically by taking small samples of thylakoid material and doing small-scale BBY preparations using Triton X-100:Chl ratios of 5:1, 10:1, 15:1, and 20:1 (w/w). A large-scale preparation was done with the mixture that gave the greatest yield with the lowest Chl *a/b* ratio. PSII RC complexes were prepared from the fresh BBY preparations according to the method of Ghanotakis et al. (1987) using their high NaCl, high Suc, octyl-glucoside solubilization protocol. We also found that the optimal amount of detergent varied between preparations (Falbel and Staehelin, 1992). The amount of detergent that gave the greatest yield of PSII and the highest Chl *a/b* ratio was selected for the large-scale preparation.

PSI RC complexes were prepared by limited Triton X-100 treatment according to the method of Mullet et al. (1980) with modifications by Bassi and Simpson (1987). The appropriate Triton X-100 concentration was determined empirically for each preparation and varied from 0.4 to 0.8%. Suc gradients were formed by freezing (at −70°C in ultracentrifuge tubes) and thawing (slowly at 4°C) 20% Suc solutions containing appropriate buffers and detergents as described by Bassi and Simpson (1987). Dodecyl Suc was substituted for dodecyl maltoside as the detergent in the second Suc gradient. Bands from the second gradient containing no LHCII were pooled and used as PSI standards.

Chl Determinations

Chl was extracted from isolated thylakoids by adding acetone to a concentration of 80% and then centrifuging to precipitate proteins. Chls *a* and *b* were quantified by determining the absorption of the supernatant at 663.6 and 646.6 nm and using the equations of Porra et al. (1989).

Electrophoresis

Native "green gel" electrophoresis was performed with some modifications to the method of Allen and Staehelin (1991). Stacking gel was omitted for some of the gels because it was found that those cast without stacking gels reproducibly gave sharper bands and fewer artifacts (Falbel, 1994; G. van der Staay, unpublished observations). Green gels 3 mm thick, 8% polyacrylamide, cast in a minigel apparatus (Hoefer, San Francisco, CA) were run at 4°C for 1.5 to 2 h at 10 mA until the free pigment was at least two-thirds of the way through the gel.

Denaturing gels were composed of 18% acrylamide/0.2% bis-acrylamide with 1,2-amino-methyl-propanediol (ammediol) as a buffer, based on the system described by Wycoff et al. (1977) and Bury (1981) and introduced previously for the analysis of thylakoid membrane proteins by our laboratory (Allen and Staehelin, 1991; Sigrist and Staehelin, 1991; Falbel and Staehelin, 1992). Gels were surrounded by electrophoresis buffer (in the Hoefer SE600 apparatus) and typically run without cooling at a constant current of between 20 and 40 mA per gel (0.75-mm-thick). A small amount of Congo red dye (Sigma) was dissolved in solubilization buffer and used as an indicator. The gels were stopped when this dye (which runs very close to the LHC apoproteins) had run at least half way through the gel. Protein bands on all denaturing gels were visualized by silver staining according to the method of Blum et al. (1987).

Electron Microscopy

Wheat and barley samples prepared for electron microscopy were cut from the upper region of a 6- to 7-d-old first leaf (approximately 0.5 to 3 cm from the tip) grown under the three different light conditions described above. Leaves were cut into small pieces and chemically fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0, vacuum-infiltrated for 15 min, and incubated for approximately 4 h. Samples were rinsed in phosphate buffer three times and then postfixed in 1% osmium tetroxide in phosphate buffer for 1 h. Then samples were rinsed in buffer, then water, dehydrated through an ethanol series, incubated in propylene oxide, infiltrated and embedded in Spurr's resin, and polymerized for 24 h at 70°C. Ultrathin sections were cut on an ultramictrotome (Leica) and stained for 30 min with aqueous uranyl acetate and 10 min with lead citrate. Samples displaying "embedding pepper" were treated with a modification of the method described by Aldrich and Mollenhauer (1986). Sections on gold slot grids were exposed to a 2% solution of periodic acid in water for 30 min. After thorough rinsing with water, the grids were poststained for 30 min with aqueous 2% uranyl acetate and 10 min with lead citrate, and then micrographs were produced (model CM10, Philips Mahwah, NJ).

RESULTS

In addition to measuring Chl *a/b* ratios by standard techniques, we used three methods to monitor the severity of the mutant phenotypes: native and denaturing gel electrophoresis and thin-section electron microscopy. Native green gels were used to provide information concerning the size of the RC-antenna complexes in the thylakoid membranes and denaturing SDS-PAGE was used to study changes in the polypeptide composition of the thylakoids, as well as to identify the composition of different CP bands on green gels. The electron microscope analysis has provided correlative morphological data about the organization of the thylakoids in situ.

Chl *b*-Deficient Mutants Are Deficient in Specific CP Complexes

The overall thylakoid protein composition of 16-d-old wild-type and CD3 wheat grown under two different different light conditions was determined by denaturing gel electrophoresis. Although many of the thylakoid proteins did not vary among the four lanes, as shown on the silver-stained gel (Fig. 1) standardized by the staining intensity of the coupling factor bands, the CD3 mutant grown under both light conditions contained significantly less LHCI and LHCII than the wild type. Additionally, CD3 wheat plants grown under high light contained much lower amounts of LHCII and LHCI polypeptides than those grown under low light. Wild-type wheat grown under high light showed only slightly less of these proteins than when grown under low light.

Figure 2 depicts the CP complexes of high-light and low-light wild-type and CD3 thylakoids as resolved on a mildly denaturing, low-ionic-strength green gel. As is typical of wild-type thylakoids, the low- and high-light lanes both demonstrated a high proportion of slowly migrating large to very large RC-antenna complexes and a relatively low proportion of smaller RC-antenna complexes and RC-core complexes. Only minor differences were seen between the low- and high-light wild-type samples. In contrast, in CD3 both the low-light and high-light lanes demonstrated a major loss of large RC-antenna complexes and a corresponding increase in smaller RC-antenna and RC-core bands compared with the wild-type samples. As predicted, the decrease in larger RC-antenna complexes was more pronounced in CD3 plants grown in high light than in those grown in low light. Finally, compared with the wild-type plants, the CD3 plants contained lower amounts of the trimeric LHCII* complexes. In parallel to the above-listed differences, the CD3 mutant plants demonstrated a light-intensity-dependent decrease in Chl content (wild type low light: 2.8 mg Chl g^{-1} fresh weight; CD3 high light: 1.1 mg Chl g^{-1} fresh weight) and an increase in the Chl *a/b* ratio (wild type low light: 4.3; CD3 high light: 14.7; Fig. 2).

To confirm the composition of the different bands, we excised the major bands from a green gel (Fig. 3A) and ran them on denaturing gels to compare each to purified PSII and PSI complexes (Fig. 3, B–D). The slowly migrating, large RC antenna green bands present in wild-type and

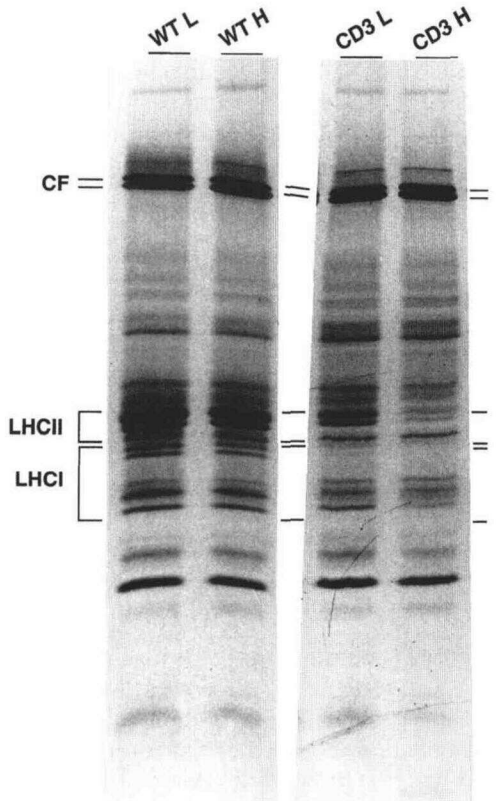

Figure 1. Denaturing gel electrophoresis of wild-type (WT) and CD3 mutant wheat grown for 16 d under high (H; approximately 1600 μmol photons m^{-2} s^{-1}) and low (L; approximately 100 μmol photons m^{-2} s^{-1}) light conditions (see "Materials and Methods"). These silver-stained gels incorporate the ammediol buffer system and contain an 18 to 25% polyacrylamide gradient. To illustrate clearly the similarities and differences among these four samples, gel was loaded by estimation such that the stained amount of coupling factor subunits were equal in all samples. Only the LHC polypeptides exhibited significant changes among these samples. CF, Coupling factor.

mutant samples contained identical components (Fig. 3B). The inner antenna molecules of PSII, CP26, and CP24 were present in the identical PSII-containing green gel bands (Fig. 3D). However in the mutant there was a notable additional green band composed of PSI RCs lacking the LHCI antennae (Fig. 3C).

Changes in the RC-Antenna Complexes Depends on the Severity of the Mutation

Not only does the severity of the growth environment influence the RC-antenna complex region of the gel, but so does the severity of the mutation and the age of the plant. Three of the homeologous allelic wheat mutants, *chlorina*-1, CD3, and *chlorina*-214 (Falbel and Staehelin, 1994), were grown for approximately 3 weeks under both the high- and low-light growth conditions. Green gels from plants grown under low-light conditions are shown in Figure 4. The samples have been ordered on the gel as to the visible severity of the phenotype and Chl content, i.e. *chlorina*-1 is the most yellow-green, followed by CD3, and

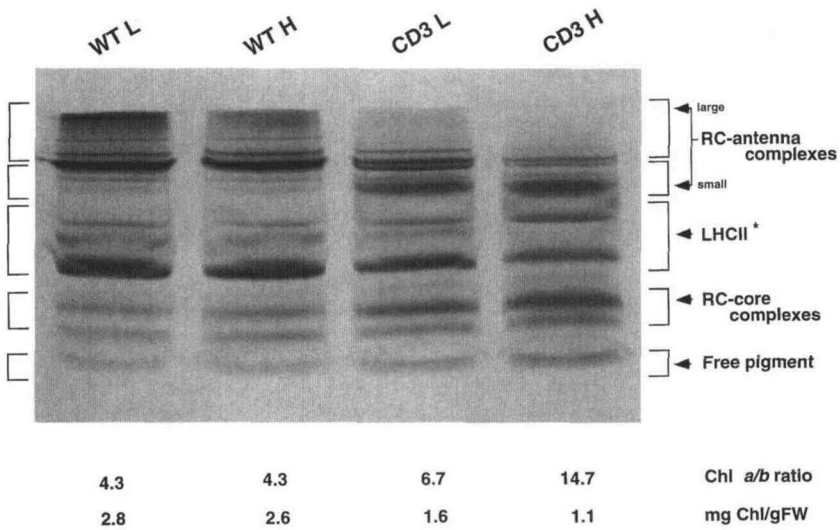

Figure 2. Low-ionic-strength green gel electrophoresis of thylakoids isolated from wild-type (WT) and CD3 mutant wheat grown under low (L) and high (H) light conditions. Chl (30–40 μg) from the thylakoid preparations used in A were analyzed on low-ionic-strength green gels. The content of Chl and the Chl *a/b* ratio of the leaf tissue used to isolate these thylakoids are shown below each lane. Chl *a/b* ratios for thylakoids isolated from these plants were: wild-type L, 3.8; wild-type H, 4.2; CD3 L, 5.9; and CD3 H, 9.0). The Chl *a/b* ratios for isolated thylakoids were slightly lower than for Chl extracted directly from whole tissue, probably because of some loss of unstacked membrane regions during thylakoid isolation.

then *chlorina*-214. As already illustrated for the high-light-grown CD3 plants (Fig. 2), the severity of the Chl deficiency is paralleled by a reduction in size of the RC-antenna complexes as evidenced by the loss of the slowly migrating green bands in the top segment of the gel and the appearance of faster-migrating and smaller RC-antenna and RC complexes. There was also an increase in the Chl *a/b* ratio from 3.7 to 4.4 (Fig. 4), but the changes were not as extreme as those shown in Figure 2, because these plants were grown under low-light conditions and were somewhat older.

Similarities in Chl *b*-Deficient Mutants of Other Species

To confirm the generality of the wheat mutant observations, we also analyzed two Chl *b*-deficient dicot mutants, the heterozygotes of the tobacco *Su* mutant (*Su*/+) and the tomato *Xa-2* mutant (*Xa-2*/+), by means of denaturing (Fig. 5) and nondenaturing electrophoresis (Falbel, 1994; data not shown). Even though the plants were grown for 4 weeks under low-light conditions, which would tend to maximize antenna development, both mutants contained significantly lower quantities of both LHCI and LHCII polypeptides than the wild types (Fig. 5), similar to the CD3 wheat mutant illustrated in Figure 1. Corresponding increases in the Chl *a/b* ratios from 2.7 for wild type to 3.3 for the mutant heterozygotes were observed for both tobacco and tomato thylakoids (Fig. 5). As was the case for the wheat mutants shown in Figures 1 and 2, the severity of the mutant phenotype for the tomato *Xa-2* mutants depended on light intensity. Chl content and Chl *a/b* ratios were measured in whole tissue for plants grown under low- and moderate-light conditions. Wild-type tomato plants grown under low light had 2.6 mg Chl g^{-1} fresh weight and a Chl *a/b* ratio of 3.0, whereas the *Xa-2*/+ heterozygote had 1.8 mg Chl g^{-1} fresh weight and a Chl *a/b* ratio of 3.8. When grown under moderate light, wild-type plants had 1.6 mg Chl g^{-1} fresh weight and a Chl *a/b* ratio of 3.0, and the *Xa-2*/+ heterozygote had 0.8 mg Chl g^{-1} fresh weight and a Chl *a/b* ratio of 6.4. Similar results for the tobacco *Su*/+ mutant were obtained and have been reported in the literature (Homann and Schmid, 1967; Koivuniemi et al., 1981; Siffel et al., 1993; Schindler et al., 1994).

Effect of Light Intensity on the Ultrastructure of Chl *b*-Deficient Mutants

To determine whether the "graded series" of wheat mutants also displayed a continuum of unstacked to stacked thylakoid ultrastructures in parallel to the differences they showed in polypeptide composition and the extent of their blocks in Chl synthesis, samples were prepared from this series of mutants for electron microscopy. Segments from the tops of 7-d-old leaves of plants grown at low, medium, or high light intensity (<100, 150, or 600 μmol photons m^{-2} s^{-1}, respectively) were chemically fixed and embedded in Spurr's low-viscosity resin according to standard methods. Figure 6 illustrates thin sections of chloroplasts taken from wild-type and mutant chloroplasts, arranged in order of severity of their morphological phenotype. Wild-type membranes showed abundant, uniform diameters and approximately parallel organized grana stacks under all light conditions (Fig. 6A). The CD3 mutant (Fig. 6B, and the chlorina-214 mutant, the other allele on chromosome 7D; not shown) also exhibited a significant amount of membrane stacking when the plants were grown under low to moderate light intensities, but their grana stacks were highly variable in size and appeared disorganized, facing in many different directions. Also there are more extensive arrays of parallel but nonstacked stroma membranes linking the grana stacks (Fig. 6B). Driscoll's *chlorina* (Fig. 6D), the most severe mutant of this series, looks identical with the *chlorina*-1 mutant (not shown), the other allele at this locus on chromosome 7A. The thylakoids of this severe mutant, grown under low- to moderate-light conditions were connected to a limited number of small, scattered grana stacks that varied in size and shape and were often connected at the ends of the parallel membrane domains. The peripheral thylakoids also tended to be more disorganized than those in the center of the chloroplasts.

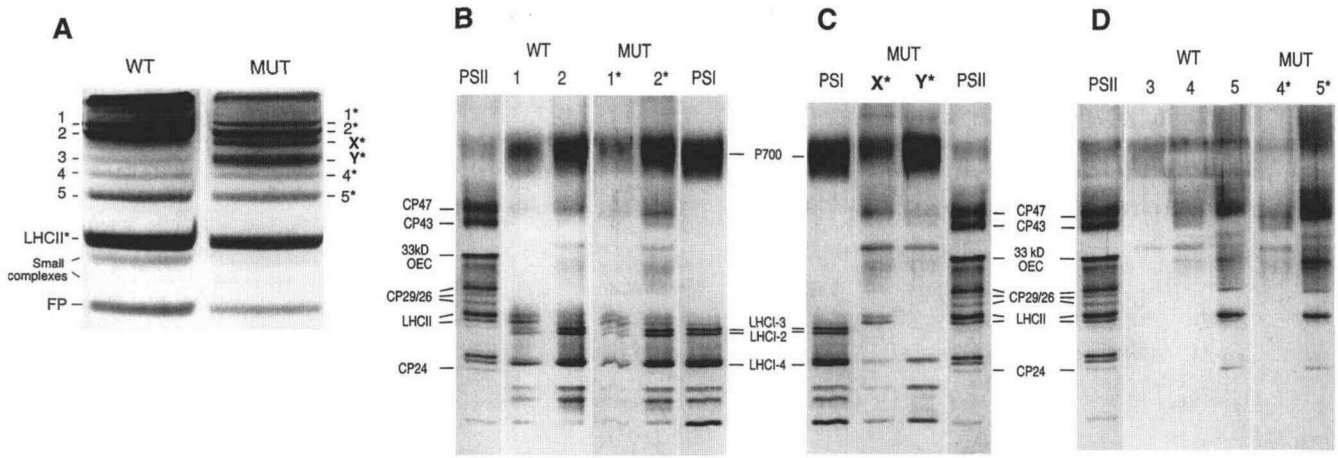

Figure 3. Protein composition of green gel bands. A, Low-ionic-strength green gel electrophoresis of thylakoids isolated from wild type and the most severe *chlorina*-1 mutant (Fig. 4). Green bands containing the large and small RC-antenna complexes were excised and run on the denaturing gels shown in B to D. The green gel was run as indicated in Figure 2 except without a stacking gel to yield sharper bands (see note in "Materials and Methods"). Green bands excised from wild type (WT) are labeled 1 to 5, and those from the mutant (MUT) are labeled 1* to 5*, with two additional bands, X* and Y*, replacing band 3*. FP, Free pigment. B to D, Silver-stained denaturing gel showing the protein composition of each of the green bands from A, alongside isolated PSI and PSII complexes from wild-type wheat. The labeled components of these purified PSI and PSII complexes were identified by immunoblot analysis using antibodies against antenna proteins and the 33-kD subunit of the PSII oxygen-evolving complex as a reference (Falbel and Staehelin, 1992; Falbel, 1994; Król et al., 1995). LHCI-2, -3, and -4 are products of the *Lhca2*, *Lhca3*, and *Lhca4* genes (Jansson et al., 1992). Proteins not labeled did not cross-react with any of our antibodies. Although our CP29 antibodies did not cross-react with any wheat proteins, CP26 antibodies do cross-react with CP29 in several species (Falbel and Staehelin, 1992). B, Composition of the two large RC-antenna bands (1 and 2) are compared with preparations of wheat PSI and PSII. These upper bands contain mostly PSI complexes but also some co-migrating PSII components. Both LHCI and LHCII are present. C, Composition of the two bands, X* and Y*, that are present in the mutant but not in the wild-type sample. There is a small amount of LHCII and CP47 apparent in the X* band, but the majority of X* and Y* consists of a PSI RC lacking LHCI. LHCI-1 was not recognized by the antibodies we used. Wild-type band 3 is clearly different from bands X* and Y* in the mutant. D, Composition of the remaining excised bands 3 to 5, 4*, and 5*, which are mainly PSII-derived. Band 5 contains the inner antenna proteins CP24 and CP29/26 (probably CP29 according to Bassi and Dainese, 1992).

To examine the light-intensity-dependent nature of these mutants, a set of CD3 mutant plants was fixed after growth for 7 d at a high light intensity (approximately 600 μE m^{-2} s^{-1}). Figure 6C shows that this growth condition greatly affects the spatial organization of the thylakoids when compared with those of CD3 plants grown under lower light (Fig. 6B). Under these high-light growth conditions, very few membrane stacks were produced. Instead, most of the thylakoids became organized in the form of long membrane sheets resembling those in the Driscoll's *chlorina* mutant grown under low- to moderate-light conditions (Fig. 6D). Some of the membrane sheets also formed extended stacked domains, but these were not converted to normal stacks. Wild-type plants never produced this type of thylakoid morphology under high-light conditions (Fig. 6A). High-light conditions increased the number of plasto-

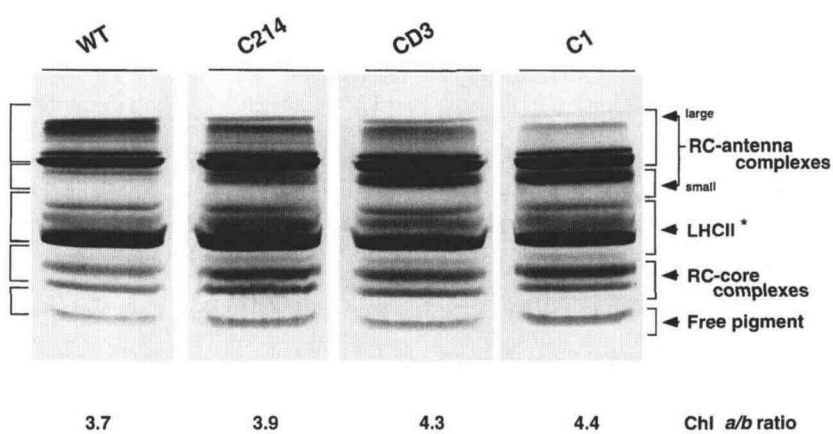

Figure 4. Low-ionic-strength green gel electrophoresis of thylakoids isolated from wild-type (WT) wheat plants compared with the allelic mutant series shown in order of severity: *chlorina*-214 (C214), CD3, and *chlorina*-1 (C1). The plants were grown under low-light conditions (approximately 100 μmol photons m^{-2} s^{-1}) for 4 weeks. Gels were loaded as described in Figure 2. The Chl *a/b* ratios of thylakoid preparations are shown.

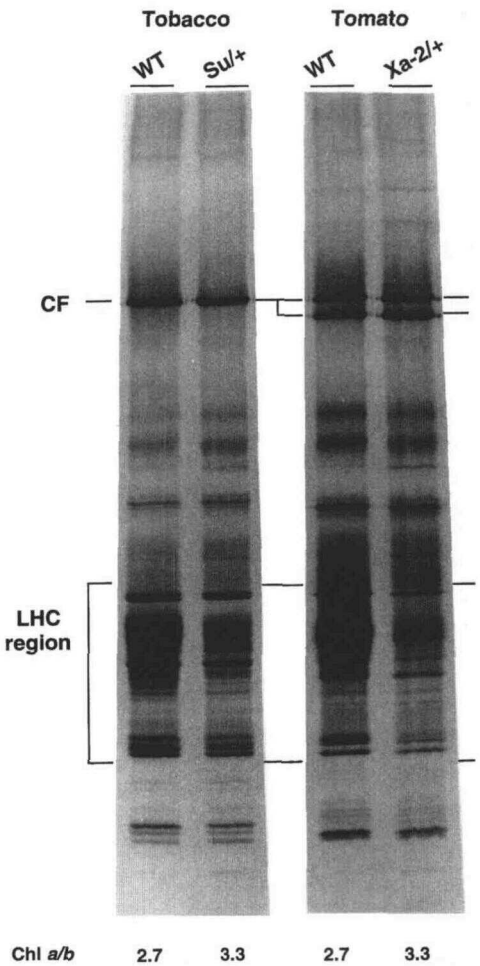

Figure 5. Denaturing gel electrophoresis of thylakoid proteins isolated from wild-type (WT) and mutant heterozygotes for the tobacco *Sulfur* mutant (*Su*/+) and the tomato *Xanthophyllous*-2 mutant (*Xa-2*/+) grown under low-light conditions (100–200 μmol photons m^{-2} s^{-1}). The gels were loaded and silver-stained as described in Figure 1. Only the LHC polypeptides exhibited significant changes among these samples. The Chl *a*/*b* ratios of thylakoid preparations are shown. The Chl content and Chl *a*/*b* ratio were also determined for leaf tissue from tomato plants grown under two different light conditions (see text), and as indicated in Figure 2, the Chl *a*/*b* ratios for the isolated thylakoids are slightly lower than those for the whole leaf tissue (see text and Fig. 2 legend). CF, Coupling factor.

globuli (lipid droplets containing tocopherols, carotenoids, and other substances; Lichtenthaler, 1968) in both wild-type and mutant plants but more so in the mutants. Driscoll's chlorina plants grown under high-light conditions became chlorotic after about 6 d and died shortly thereafter.

DISCUSSION

The long-term goal of our research is to determine how and to what extent Chl biosynthesis regulates the morphogenesis of thylakoid membranes. In this study we have examined the relationship between growth, light intensity, and severity of the biochemical and ultrastructural phenotype of a graded series of wheat mutants with blocks in the Chl synthesis pathway. Our main findings are that the apparent severity of the phenotype for any given mutant increases with the light intensity under which the plant is grown and that changes in light intensity can be used to make different mutants exhibit the same phenotype.

The Phenotypic Spectrum of Chl *b*-Deficient Mutants

All of the Chl *b*-deficient mutants that we have analyzed to date have a block in the Chl synthesis pathway (most frequently at protoporphyrin IX; Falbel and Staehelin, 1996) and express the following characteristics in a light-intensity-dependent manner (Falbel and Staehelin, 1996, and refs. therein): (a) a reduced content of Chl, (b) a preferential loss of Chl *b*, (c) a significant reduction in light-harvesting CP complexes, (d) the formation of grana-deficient thylakoid membranes (Schmid et al., 1966; Hopkins et al., 1980; Allen et al., 1988; Greene et al., 1988a), and (e) the accumulation of a larger pool of xanthophyll cycle pigments (Falbel et al., 1994; Schindler et al., 1994).

The reduced Chl content (Fig. 2) of Chl *b*-deficient wheat mutants has been traced to a slower accumulation of Chl, with the rate of accumulation being inversely proportional to the severity of the mutant phenotype (Falbel and Staehelin, 1994). That study also showed that the mutants with the most severe phenotypes accumulate the most Chl precursors, which increases their potential for poisoning by the photosensitization of these precursors. Upon excitation by a photon, protoporphyrin IX molecules dissipate their energy through free radical formation, resulting in oxidative photodamage of pigment molecules, proteins, and lipids (Jacobs and Jacobs, 1993; Smith et al., 1993). We also postulated that the increased severity of the phenotypes of Chl *b*-deficient mutants under high light intensities is due to both increased photobleaching of Chl precursors and to the inability of the limited Chl synthesis pathway to keep up with increased damage to the Chl pool under high illumination, because the potential capacity for photoprotection may be insufficient to dissipate all of the excess excitation energy (Falbel et al., 1994). This greater strain on the Chl synthesis pathway further amplifies the inhibition of the Chl *b* synthesis pathway, which seems to use leftover Chl *a* molecules from the former pathway as a substrate. This scenario would explain the drastically increased Chl *a*/*b* ratios of the Chl *b*-deficient mutants grown at high light intensities (Fig. 2). These high-light effects are most pronounced in young plants, in which the demand on Chl synthesis by the assembling photosynthetic apparatus of the thylakoid membrane is the greatest (data not shown).

Light-Intensity-Dependent Interconversion of Chl *b*-Deficient Mutant Phenotypes

Because Chl *b* is found only in light-harvesting CPs, limiting the supply of Chl *b* affects only antenna complexes that need both Chl *a* and Chl *b* for stability. Chl *a*/*b* proteins that lack a full complement of Chl *b* molecules are unstable and are degraded rapidly (Bennett, 1981; Bellemare et al.,

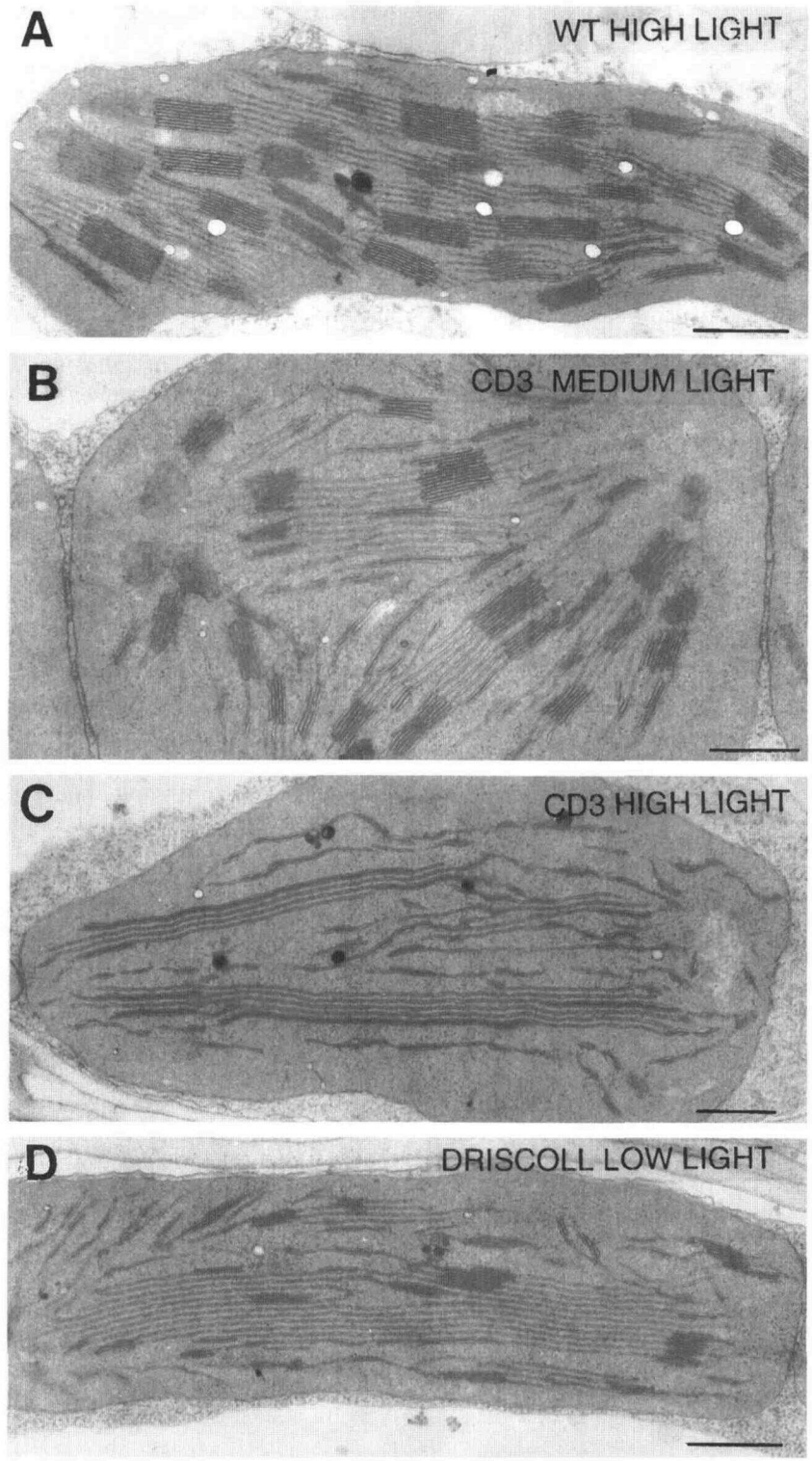

Figure 6. Light-intensity-dependent modulation of chloroplast ultrastructure. A, Wild-type (WT) chloroplasts have abundant, well-ordered stacks at all light intensities. These plants were grown at high light intensity (600 μmol photons m^{-2} s^{-1}). B, CD3 mutant wheat grown at moderate light intensity (150 μmol m^{-2} s^{-1}) has an intermediate amount of membrane stacking but with less order than wild type. C, CD3 mutant wheat grown at high light intensity (600 μmol m^{-2} s^{-1}) has few or no membrane stacks and only occasional long, appressed areas among the long, parallel, unstacked membranes. D, A similar morphology to C, in which there are very few peripheral membrane stacks. The CD3 plants used for this fixation were the same plants used for carotenoid determinations described by Falbel et al. (1994). D, Driscoll's *chlorina* mutant grown under very low light conditions (30 μmol m^{-2} s^{-1}). It is the most severe allele of the CD3 mutant. Abundant plastoglobulae are observed in A and C for the plants grown under high light conditions. All plants (A–D) were 7 d old. Bar equals 0.5 μm.

1982). Conversely, when the Chl *b* level in a Chl *b*-deficient mutant is experimentally increased, e.g. by applying chloramphenicol to inhibit the synthesis of RC proteins that bind only Chl *a*, the increase in Chl *b* is matched by an increase in LHCs (Duysen et al., 1985, 1987). Also, greening wild-type barley plants can be treated with the porphyrin precursor 5-aminolevulinic acid to increase the supply of Chl in the greening experiment. After this treatment the amount of Chl *b* increased more than the amount of Chl *a*, and the amount of LHC proteins increased preferentially over the RC polypeptides (Tanaka et al., 1993, 1994).

In this study, we have extended these observations to show not only a light-intensity-dependent loss of PSI and PSII antenna polypeptides (Fig. 1) but also a loss of Chl *b*-containing antenna complexes. These latter changes are evidenced in our green gels by the loss of large RC-antenna complexes (Figs. 2–4), which appear to be related to the large "photosynthetic units" measured by spectrophotometric means (Ghirardi and Melis, 1988; Greene et al., 1988b). Losses in LHCs in Chl *b*-deficient mutants have also been shown to lead to a reduction in size of the freeze-fracture particle equivalents of RC-LHC and LHCII complexes (Allen et al., 1988; Greene et al., 1988a; Knoetzel and Simpson, 1991).

When the green gels shown in Figures 2 and 4 are compared, it can also be seen that, by increasing the light intensity under which the CD3 wheat mutant is grown, its green gel pattern can be made to resemble the pattern of the more severe wheat chlorina-1 mutant grown at a lower light intensity. This finding suggests that the rate of Chl accumulation and the growth light intensity are the principal determinants of the biochemical phenotype of Chl *b*-deficient mutants.

Paralleling these biochemical changes are changes in thylakoid architecture. All of the Chl *b*-deficient mutant morphologies illustrated in Figure 6, as well as many intermediate architectures, have already been demonstrated in the literature (Hopkins et al., 1980; Allen et al., 1988; Greene et al., 1988a; Henningsen et al., 1993). However, these diverse morphological phenotypes have been difficult to explain because of the incomparable growth conditions. Only by growing a graded series of allelic wheat mutants under several identical conditions (light intensity, temperature, and length of time) have we been able to relate the most severely blocked mutants to the mutants with the least severe blocks and comprehend how these different mutants give rise to a continuum of mutant phenotypes. The plasticity of the CD3 mutant phenotype suggested to us the interrelatedness of the morphological phenotypes in both (a) greening experiments at different light intensities (Allen et al., 1988) and (b) depending on gene dosage of the CD3 mutation (Freeman et al., 1987). In this study, the CD3 mutant could interconvert from a severe, unstacked Driscoll mutant type of morphology (when grown under high light, Fig. 6C), reminiscent of the *xantha* mutants of barley with nearly complete blocks in Chl biosynthesis (von Wettstein et al., 1971; Henningsen et al., 1993) to a nearly wild-type morphology (when grown under low to moderate light). Intermediate morphological phenotypes could be generated with various combinations of mutants and growth conditions (not shown).

This family of mutants with partial blocks in Chl biosynthesis grown at different light intensities and for differing periods of time could therefore be modeled with a simple "supply and demand" dynamic of Chl accumulation and photobleaching during both chloroplast development in young leaves and in the maintenance of chloroplasts in mature leaves. Mutant chloroplasts reach a different steady-state level of Chl, Chl-binding proteins, and membrane stacks, depending on what supply the pathway can provide to meet the demand of the environment, and take longer to reach this steady state than wild-type plants. Indeed, studies of the Chl content in the *Su*/+ tobacco heterozygote demonstrated the existence of a "slowdown" of chloroplast development; the mutant reached its steady-state level of pigment later than wild-type chloroplasts (Siffel et al., 1993; Schindler et al., 1994).

Hierarchical Binding of Chl to Chl-Apoproteins Appears to Control Thylakoid Morphology

When the Chl *b*-deficient OY-YG mutant of maize is grown under conditions of high light it exhibits a differential reduction in the accumulation of its Chl *b*-containing antenna complexes, with the peripheral antenna proteins (trimeric LHCII) more affected than the inner antenna protein (CP29; Greene et al., 1988a). Similarly, Allen et al. (1988) found that the inner antenna proteins (CP29 and CP24) of the CD3 wheat mutant in greening experiments were less affected than the peripheral trimeric LHCII*. This hierarchy corresponds to the well-documented order of appearance of these Chl-binding proteins during development: first the RC components of PSI and PSII, followed by the minor/inner antenna proteins of PSII (CP29, CP26, and CP24), and finally LHCI and peripheral (trimeric) LHCII (Dreyfuss and Thornber, 1994; Sigrist and Staehelin, 1994; Paulsen, 1995, and refs. therein). Biochemical studies of RC complexes from wild-type, mutant, and developing plants in a variety of species have led to numerous models of the RCs that place the minor LHCs as links between the RC cores and peripheral antennae (reviewed by Jansson, 1994; Thornber et al., 1994). Intrinsic to these models is the notion that during development the photosynthetic unit increases through the addition of antenna complexes outward from a functioning RC core and that mutants deficient in Chl *b* never finish this process and lack the peripheral antennae.

In this scheme, the greater stability of the inner versus the peripheral antenna complexes in the Chl *b*-deficient thylakoids may have several origins: most inner antenna apoproteins bind less Chl *b* and are bound to RC core proteins, which bind only Chl *a*. In contrast, stable folding of the peripheral LHCII apoproteins requires both Chl *a* and Chl *b* (Plumley and Schmidt, 1987) and since the proteins form homotrimer complexes (Kühlbrandt et al., 1994), little additional folding stability can be gained from intercomplex protein-protein interactions.

A model for the organization of antenna systems should be consistent with the results from all types of mutants

lacking Chl *b*. In the introduction, we stated that it is critical to distinguish the Chl *b*-deficient mutants from the mutants containing no Chl *b* at all. Even though these two classes of mutants have a reduction in the amount of peripheral antenna proteins and some other common features (discussed by Falbel et al., 1994), we stress that the assembly and developmental process of these two thylakoid membrane systems differ greatly because of the critical reduction in Chl *a* in addition to the lack of Chl *b* in the Chl *b*-deficient mutants. In both types of mutants, a steady-state thylakoid composition is reached with a reduced pool of Chl *b*-binding proteins, but the paths taken to reach them are indeed quite different. For this reason we postulate that it is not correct to think of the mutants with no Chl *b* as a more severe form of the *b*-deficient mutants, as is commonly done in the field. In the Chl *b*-less barley mutant *chlorina* f2 the type III LHCII proteins are present at wild-type levels and are capable of binding only Chl *a* (Harrison and Melis, 1992; Harrison et al., 1993; Król et al., 1995), consistent with the biochemical data, suggesting that the type III LHCII (Lhcb3) is bound directly to the RC cores (Bassi and Dainese, 1992). However, in the same barley mutant the inner antenna protein CP24 is curiously absent (Król et al., 1995), whereas it is present in the Chl *b*-deficient mutants we have analyzed. One could speculate that, without the pool of trimeric peripheral LHCII that is normally linked to the RC through CP24, this linker antenna is degraded because there is no need for such a link, even though CP24 may be capable of binding Chl *a* alone in vitro (preliminary CP24 reconstitution results of S. Hobe, R.B. Klögsen, and H. Paulsen, cited by Paulsen, 1995). Likewise, other inner antenna complexes may be more stable in the presence of Chl *a* alone because they may have other roles, such as in the regulation of the xanthophyll cycle (reviewed by Paulsen, 1995).

The allelic series of Chl *b*-deficient wheat mutants has allowed us to clarify the interplay between pigment and protein accumulation in thylakoid morphogenesis. For Chl *b*-less mutants the interplay works in only one direction: the lack of Chl *b* affects the abundance of only certain proteins and protein complexes. Król et al. (1995) found that the levels of particular antenna proteins in the Chl *b*-less *chlorina f2* mutant did not correlate with Chl *b* content, as it seemed to do in our studies. They found that only certain antenna proteins were affected by the lack of Chl *b* and others were expressed at wild-type levels. In the Chl *b*-deficient mutants, all of the Chl *b*-binding proteins are affected but to different extents depending on the severity of the mutation and the environment. The interplay between pigment and protein accumulation works in both directions for the *b*-deficient mutants: the shortage of total Chl (due to partial blocks in the Chl synthesis pathway) causes the redirection of more Chl toward the RCs rather than toward the Chl *b* synthesis pathway (proteins affect pigment), and the lack of Chl *b* in turn affects the abundance of certain proteins (pigment affects proteins). Over time and under mild growth conditions, the result of this block can be substantially overcome in the *b*-deficient mutant plants, as mentioned above. The mutants with no Chl *b* exhibit much less plasticity in their phenotype and no amount of time will increase the pool of Chl *b*.

Summary and Perspective

Previously we suggested that a partial block at any step of the Chl synthesis pathway would have the same effect on chloroplast development (Falbel and Staehelin, 1996), whether the block was through a mutation or through environmental effects. Limiting the light-requiring photoreduction of protochlorophyllide to chlorophyllide in Chl biosynthesis by manipulating the light conditions (greening under continuous far-red light, De Greef et al., 1971; or intermittent light, reviewed by Paulsen, 1995) also has been shown to generate some thylakoid membranes deficient in Chl *b* and LHCs and exhibiting similar unstacked morphological phenotypes to those shown in Figure 6, C and D, for wheat CD3 and Driscoll mutants. Environmental manipulations such as these may also exert other indirect effects on the Chl synthesis pathway and chloroplast development via photoperception and signal transduction processes that are not well understood under these conditions. Similarly, some of the blocks in the pathway that we and others have observed for Chl-deficient mutants may be a result of either direct or indirect effects of those mutations. Our theory states that the steady-state level of Chl and Chl-binding proteins that a plant maintains in a certain light environment could be determined chiefly by the fine-tuning of various points of the Chl synthesis pathway. Therefore, by regulating the rate of Chl synthesis, a plant could control to a very large extent the assembly of its whole photosynthetic apparatus. Future work is required to determine whether this is the case and, as we have suggested elsewhere, to determine to what extent the regulation takes place at the first committed step of the pathway, the Mg-chelatase step (Falbel and Staehelin, 1994, 1996). Our hypothesis that Chl *b* is somehow synthesized from leftover Chl *a* must remain abstract until the oxidase catalyzing the conversion of the methyl group to the formyl group (Porra et al., 1994) is isolated and one can begin to study the regulatory associations of this enzyme with the rest of the Chl synthesis pathway and the Chl *a*/*b*-binding apoproteins.

ACKNOWLEDGMENTS

We would like to thank all of the suppliers of the mutant seeds listed in "Materials and Methods" and Dr. Stefan Jansson for the LHCI antibodies. Ms. Victoria Krebs kindly provided assistance with embedding of many samples for electron microscopy.

Received January 16, 1996; accepted June 27, 1996.

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